PHOTOSYNTHETIC PHOSPHORYLATION BY CHLOROPLASTS OF OATS IN RELATION TO CROWN RUST INFECTION

By

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ABBREVIATIONS AND SYMBOLS

The abbreviations and symbols used in this paper are those recommended by the Committee on Form and Style of the Conference of Biological Editors (15); those used for chemical compounds are: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; Pi, orthophosphate; FMN, flavin mononucleotide; PMS, phenazine methosulfate; CMU, 3-(p-chlorophenyl)-1,1-dimethylurea; Tris, tris(hydroxymethyl)aminomethane; Tween 20, trade name for a polyoxyalkylene derivative of sorbitan monolaurate, a wetting agent manufactured by Atlas Powder Company, Wilmington, Delaware.

INTRODUCTION

The way in which obligate parasites such as the plant rusts obtain nourishment from their hosts is an intriguing problem, not only in its own right, but also in furthering the understanding of disease relationships. V. M. Cutter (in collaboration with H. Hotson) was the only one who has reported a successful adaptation of a rust organism to artificial media (17). In a paper presented in 1951 (16), he advanced the hypothesis that obligate parasites require phosphorylated intermediates from their hosts and suggested that either nucleotides or sugar phosphates might meet these requirements. His induction, based on previous work of others together with his own observations, makes a reasonable argument. It was this paper that stimulated the work in this thesis.

If Cutter's hypothesis is correct, one would expect the presence of obligate parasites to alter carbohydrate metabolism in plants. The most obvious change that occurs in susceptible plants infected with rust or mildew is a striking increase in rates of respiration (39, 52). The hexose monophosphate shunt seems to participate more in the respiration of rusted plants than it does in healthy plants (18, 47), and rates of photosynthesis are generally reduced after infections by the rusts and mildews (2, 33, 45, 46). Changes in levels of phosphate compounds at infection sites (23, 26) have implicated phosphate metabolism as playing an important part in the growth of obligate parasites. At the present time, none of these observed changes can be directly correlated with the ability of the parasite to obtain food from its host. However, when photosynthesis in infected plants is stopped by placing the plants in the dark or in a carbon dioxide-free atmosphere, the development of the parasite is also stopped. But if an external carbohydrate source is made available to these plants, the parasite continues to develop normally (16).

If the observations just mentioned are to be brought together and investigated more thoroughly, more must be learned about the relationships between obligate parasitism, carbohydrate metabolism, and phosphorylated intermediates. One way to do this is to assume that Cutter's hypothesis is correct and that, more specifically, some of the intermediate products of photosynthesis are important in the nutrition of the obligate parasites. This leads to a further assumption that the photosynthetic reactions in the susceptible plant would respond differently to infection than those in the resistant plant.

Therefore, I hypothesize that photosynthetic phosphorylation is stimulated in susceptible rust-infected plants, whereas it is either unchanged or depressed in resistant infected plants. Photophosphorylation produces the first stable intermediate of photosynthesis, viz. ATP, and the process may be studied by using isolated chloroplasts. A study of the rates of photophosphorylation in chloroplasts under the proper conditions should reveal whether the parasite interferes with the light reaction so that ATP production is either stimulated or depressed.

MATERIALS AND METHODS

Biological material

Host and parasite.--Two varieties of oats, *Avena* sp., were used together with Race 290 of the crown rust fungus, *Puccinia coronata* Cda. f. sp. *avenae*. Floriland (reaction type of 3 - 4)[1] was used as the susceptible variety. The resistant variety, C.I. 7233,[2] gives a reaction type of 0 - 1. Seed of Floriland oats were obtained from the Florida Agricultural Experiment Station, Gainesville, and seed of C.I. 7233 from M. D. Simons, Iowa State University, Ames. Race 290 was maintained by uredial transfers on mature Floriland plants in the greenhouse. Inoculations were made by wetting the plants with 0.01 per cent Tween 20 in tap water and then dusting on the spores in a 1:4 mixture with talc. Plants were kept in a polyethylene moist chamber for 12 - 24 hours. The urediospores were collected with a vacuum-type spore collector and stored at 5° C. Viability was usually maintained for two to three weeks. The race was checked periodically for contamination by testing it on a set of five differentials. After a suspected contamination in August, 1962, the stock was renewed with a sample of the original collection from Quincy, Florida.

Experimental plants.--Only the first seedling leaves of young plants 8 - 20 days old were used as experimental material. The two varieties are unlike in their growth habits since C.I. 7233 germinates more rapidly, produces a longer and narrower leaf, and subsequently tends to lodge more easily than Floriland. But since there was no indication that the chloroplast preparations from each variety were basically different, Floriland was grown for most of the preliminary experiments.

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[1] Standard reaction type classification for cereal rusts: "0" - necrotic flecks, "1" - minute uredia, . . . "4" - large uredia.

[2] C.I. refers to accession numbers used by the Cereal Crops Research Branch, U. S. Dept. Agr.

Plants used in the early experiments were grown in flats of methyl bromide treated soil in the greenhouse. An evaporator-type cooling system with a steady current of air pulled through moist pads maintained a temperature range of 24 - 32° C at the level of the plants. The relative humidity usually varied from 50 - 100 per cent, but was near 90 per cent most of the time. Later, plants were grown under controlled conditions. They were planted in flats of vermiculite in the greenhouse and watered with tap water for three to four days. When the first leaves emerged from the coleoptiles, the flats were moved to a room regulated for 22° C in which the maximum temperature variation was 20 - 25° C and the relative humidity variation was 50 - 70 per cent. Light was supplied by eight 40-watt warmwhite fluorescent lamps (emission maximum: 660 mμ) supplemented with three 50-watt incandescent bulbs to give 700 - 800 foot-candles illumination[3] at midway the height of the plants. Because of possible damage to the seedlings from ultraviolet radiation emitted near the ends of the fluorescent lamps, the light was filtered through single thickness glass. The light period was maintained at 14 hours out of 24 by means of an automatic timer. All flats were partitioned into two compartments so that both varieties were grown side by side. They were supplied with Hoagland's solution in deionized water once daily. All of the comparative studies with healthy and diseased plants and some of the preliminary experiments were carried out with plants grown under these controlled conditions.

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[3] Illumination was measured with a direct reading Weston incident light meter.

Inoculation of the experimental plants was the same as that described for the nurse plants except that a weighed quantity of urediospores was used. Variation in the intensity of infections with different batches of spores was an occasional problem. In the controlled environment the fleck stage of infection appeared on both varieties five days after inoculation, and sporulation began on Floriland seven to eight days after inoculation. The seedlings were inoculated nine to thirteen days after planting and were usually harvested six days later.

Reagents

Flavin mononucleotide (sodium salt), vitamin K_5 (4-amino-2-methyl-1-naphthol hydrochloride), phenazine methosulfate (N-methylphenazonium methosulfate), adenosine monophosphate, and adenosine triphosphate were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. The adenosine diphosphate (trisodium salt) used in the first preliminary experiments also came from Nutritional Biochemicals, but that (ADP, monosodium salt) used in the rest of the work was produced by Pabst Laboratories and purchased from California Corporation for Biochemical Research, Los Angeles 63, California. The 3-(*p*-chlorophenyl)-1,1-dimethylurea (CMU) was generously supplied by R. W. Varner, E. I. duPont de Nemours & Co., Wilmington 98, Delaware. Since the commercial preparations of ADP were found to contain varying amounts of AMP, the percentage of the pure product had to be determined. A calculated number of micromoles of the commercial preparation was added to a complete reaction mixture containing a quantity of chloroplasts in excess of that needed to convert all of the added ADP into ATP. Incubation was carried out in the light, and the number of micromoles of Pi esterified was determined. The ratio of the actual amount of ADP used to the calculated amount added gave the purity of the product.

All solutions used in the preparation and incubation of the chloroplasts were made with glass redistilled water.

Preparation of chloroplasts

The conditions for isolation and reaction of the chloroplasts were worked out during the experiments with chloroplasts from healthy plants. The procedures described in the present section are the ones that proved to be the most satisfactory. All operations with leaves and chloroplasts were carried out on ice with precooled glassware and solutions so that the temperature was kept close to 0° C.

Leaves were harvested by cutting the seedlings above the stems and immersing immediately in ice water. The leaves were quickly shaken dry, weighed, rinsed with distilled water, and shaken dry again. They were then cut into lengths of 0.5 cm and dropped into 20 ml of extracting solution. This solution (10) contained 0.4 M sucrose, 0.05 M Tris, 0.01 M NaCl, and 0.02 M ascorbic acid. The pH was adjusted to 7.5 with NaOH. The extracting solution was used also for the washing and the final suspension of the chloroplasts. Extraction was carried out in a 100-ml container with a VirTis "23" homogenizer adjusted to a setting of 70 on its rheostat and run for two periods of five seconds each. Four g of leaves were homogenized in this way each time. When more material was needed, the procedure was duplicated with another 4 g batch. The homogenate was strained through eight layers of cheesecloth and then poured into a 20-ml cellulose nitrate centrifuge tube which was encased in ice within an 80-ml tube. A Servall angle centrifuge, type SP, was used inside of a refrigerator. The homogenate from infected leaves and their controls was first centrifuged at 500 x *g* for two minutes; the supernatant fraction was saved and centrifuged at 1000 x *g* for ten minutes; the pellet was resuspended in 20 ml fresh solution and washed by centrifuging again at 1000 x *g* for ten minutes. The final pellet was saved and resuspended in 1 - 2 ml fresh solution.

Resuspending the pellet contents uniformly was difficult because of their mucilaginous consistency. The most suitable method tried involved the use of a glass stirring rod tipped with a small piece of absorbent cotton (7).

The chloroplast content of the final suspension was adjusted by determining the chlorophyll concentration in an aliquot and then adding enough extracting solution to give the desired dilution. The usual adjustment was to 0.4 mg of chlorophyll per ml of suspension. By this extraction procedure, 4 g of leaves yielded 0.8 - 1.0 mg of chlorophyll or enough chloroplasts to make up a little more than 2 ml of suspension.

In some of the preliminary experiments the chlorophyll concentration was not adjusted. It was found later, however, that the rate of phosphorylation in terms of Pi uptake per mg chlorophyll per hour increased with the increase in chlorophyll concentration; therefore, a correction had to be applied to some of the data so that they could be compared between experiments. The correction factor was based on the slope of the regression line calculated from the plot of the rate of Pi uptake against chlorophyll concentration (Figure 1). This

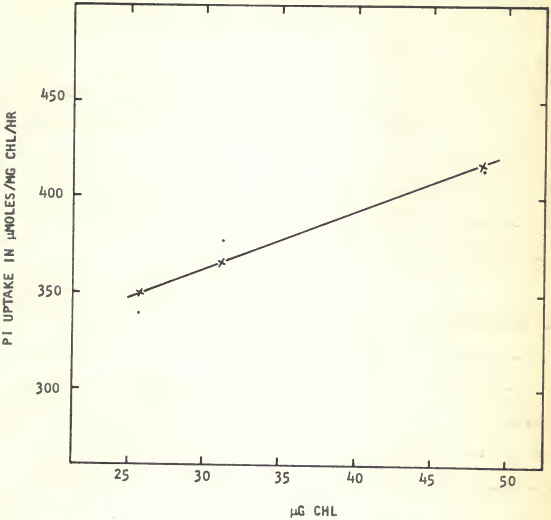

Fig. 1. Regression line calculated for the plot of rate of Pi uptake against chlorophyll concentration and used as a correction curve for some of the experiments with chloroplasts from healthy plants. The linear relationship does not hold below 20 and above 50 µg of chlorophyll. Results of the experiment from which these data were calculated are shown in Fig. 6, curve a. Reaction time, 10 min. Illumination, 4000 f-c. Total vol, 2 ml.

slope varied between some experiments, and it was found to be a function of the volume of the reaction medium as well as of chlorophyll concentration. Therefore, the correction was used only with those experiments in which the reaction mixture was 2 ml, the original concentration of chlorophyll was within the limits of the regression curve, and environmental factors such as incubation time and illumination were not varied. As used, the correction was considered accurate for standardizing the chlorophyll to 0.04 mg per ml of reaction mixture.

Reaction conditions

The composition of the reaction medium for photophosphorylation was modified from the procedures used by Arnon (7) and Avron (10) and was altered somewhat during the course of the experimentation. The standard mixture described below was established by the time the preliminary experiments were completed. The concentrations of the components are not highly critical except that there must be a sufficient amount of ADP and Pi and an optimal amount of cofactor. $MgCl_2$ concentration may be varied considerably under the conditions used here without any effect on chloroplast activity, although the optimal Mg^{++}/ADP ratio is apparently 2/1 (40).

A sufficient amount of reaction medium for a given experiment was made up without the chloroplast suspension and adjusted to pH 7.5 with a Coleman Model 28 meter. It was then pipetted into the main compartments of conical Warburg vessels containing 0.1 ml of 2N perchloric acid in their single sidearms. The vessels were then chilled in a pan of ice before 0.1 ml of chloroplast suspension was added to each. Each main compartment, then, contained 1.0 ml of the complete reaction mixture made up of the following components (figures in micromoles except for chlorophyll): sucrose, 40; Tris, 25; K_2HPO_4, 6; ascorbate, 10; $MgCl_2$, 8; NaCl, 6; PMS, 0.05; ADP(Na), 4.6; NaOH to adjust pH; and chloroplasts containing approximately 0.04 mg chlorophyll. The ADP was not dissolved and the ascorbic acid was not neutralized until they were put into the reaction medium. If additions were made to the mixture, they were pipetted into the vessels individually in 0.1 ml amounts before the chloroplasts were added, but the original reaction mixture was adjusted so that the total volume was always 1.0 ml. In earlier experiments, the complete mixture was made up to 2.0 ml; this offered no advantage and had the disadvantage of making a longer light path. The zero time controls were identical to the others except that they were made up in test tubes. All reactions were run in duplicate.

A Bronwill Warburg apparatus with a circular plexiglass bath chamber was used for incubation. The bath temperature was maintained at 15 ± 0.1° C by pumping ice water through a coil immersed in the bath. The light was furnished by fourteen 40-watt incandescent bulbs arranged in a circle 5.5 cm directly beneath the bottoms of the vessels. These lights provided an illumination of 2800 foot-candles at the level of the vessels. Additional lights were supplied by using six 150-watt floodlamps placed at a distance of 32 cm from the vessels. When both sets of lights were used together, the illumination was 4200 foot-candles. But since the luminous intensity of the floodlamps was not uniform, this greater illumination was unsatisfactory and was not used in the later experiments.

The vessels were quickly attached to the manometers, placed in position, and flushed with dry nitrogen for 2 1/2 minutes at a rate sufficient to pass 1 liter through each vessel. Nitrogen was used in all experiments except those specifically noted. The stopcocks were closed and the reaction started by turning on the lights and starting the shaker. At this time the perchloric acid was added to the zero time controls which were still in the ice bath. There was no measurable reaction from the time the flasks were removed from the ice until the lights were turned on. Dark controls were run in vessels covered with aluminum foil. At the end of incubation (usual time, 20 minutes) the lights were turned off, the perchloric acid was dumped from the sidearms, and the vessels were returned to an ice bath. The contents were mixed thoroughly, poured into tubes, and centrifuged at 800 x *g* for ten minutes. The resulting supernatant fraction was kept at 0° C (or -17° C if overnight) for analysis.

Analyses

Bill Heller: How to filter?

Chlorophyll.--Chlorophyll was determined by the second method of Arnon (4). Aliquots of 0.1 ml were taken from the chloroplast suspension, diluted to 10 ml with 80 per cent aqueous acetone, filtered, and read at 652 mμ on a Spectronic 20 spectrophotometer. The concentration was calculated on the basis of the absorption coefficient given by Arnon for a combination of chlorophylls *a* and *b*.

Inorganic phosphate.--The esterification of Pi was determined by subtracting the amount of Pi in the reaction mixture after incubation from the initial amount.

In selecting the best method for determining the Pi in a particular biological system, one is faced with two major problems: interference from other compounds and hydrolysis of labile phosphate esters during the analysis. The Lowry and Lopez method (34), designed to minimize hydrolysis of phosphate esters by making use of a low acidity, was found to be unusable because of interference. The technique devised by Berenblum and Chain (13) has proved to be a satisfactory solution for both problems. It employs the usual procedure of forming phosphomolybdic acid which is then reduced to a blue complex, the intensity of which is proportional to the amount of Pi present in the solution. But the uniqueness of the technique is its two-phase system in which the phosphomolybdic acid is extracted with an organic solvent while the phosphate esters and interfering substances are left in the aqueous phase. In the original procedure, the phosphomolybdic acid was extracted with isobutanol and was then reduced with stannous chloride. The process was complicated by the necessity of washing the isobutanol, but it was later simplified by Martin and Doty (38) who used isobutanol-benzene as the organic phase. Further modifications were made in the present work by using butyl acetate as the organic solvent (solubility in water is 0.5 g per 100 g at 25° C vs. 9.5 g per 100 g at 18° C for isobutanol), ascorbate as the reducing agent, and phase separation after reduction instead of before.

The procedure was carried out as follows: 3 ml n-butyl acetate, 0.05 ml of the acidified supernatant fraction, and 3 ml of ascorbate-molybdate reagent (4 parts 0.25 per cent ascorbic acid in water mixed just before using with 1 part 5 per cent ammonium molybdate in 6 N sulfuric acid) were added in order to a test tube. The mixture was allowed to sit for one minute and was then shaken for 15 seconds. After separation a 2 ml aliquot of the butyl acetate (upper) phase containing the blue phosphomolybdate complex was pipetted into a clean test tube, 2 ml of 95 per cent ethanol added, and the contents mixed. Absorbency readings were made at 750 mμ with the Spectronic 20. Standard curves were prepared each time with 0.1, 0.2, and 0.3 μmoles of K_2HPO_4 substituted for the supernatant fraction.

In view of the difficulties involved in developing dependable Pi analyses, it might be well to say more about the chemistry of the reaction. It has been shown that molybdic acid may be reduced by many compounds but that this reaction is greatly accelerated by the addition of Pi and is dependent upon the acidity of the solution (12).

In the two-phase system the phosphomolybdic acid may be extracted from the aqueous phase either before or after it is reduced. It was found necessary to extract after reduction in the procedure described in this paper because the amount of ascorbate in the chloroplast supernatant fraction was enough to initiate reduction as soon as molybdate was added. Therefore, the ascorbic acid reagent was added with the molybdate so that reduction would go to completion.

The rate and extent of the blue phosphomolybdate development were found to be dependent largely upon the acidity, temperature, and volume of the aqueous phase. An increase in the hydrogen ion concentration delays color development when ascorbate is used as the reductant, although this delay is not great until the hydrogen ion concentration is over 1.4 N. An increase in temperature causes a more rapid color development. As a result of these opposing conditions, a hydrogen ion concentration of 1 N may be used satisfactorily if the reagents are at temperatures of 10 - 20° C but a concentration of 1.2 N should be used at temperatures of 20 - 30° C. The volume of the aqueous phase should be equal to or greater than the volume of the organic phase. A smaller aqueous phase results in a delayed development of the blue phosphomolybdate. The concentrations of the reactants may be varied considerably. Ammonium molybdate and ascorbic acid are satisfactory at 0.4 - 2.0 per cent and 0.1 - 0.4 per cent, respectively. In these experiments the molybdate was 1.0 per cent and the ascorbate 0.2 per cent of the aqueous phase.

The extraction of phosphomolybdic acid by butyl acetate is primarily dependent on the hydrogen ion concentration of the aqueous phase. It may be seen in Fig. 2 that the reduced phosphomolybdic acid remains in the aqueous phase when there are no hydrogen ions in solution except those from the ascorbic acid but that it moves entirely into the butyl acetate phase when the acidity is increased by the addition of sulfuric acid. The quantitative relationship between the hydrogen ion concentration and the amount of reduced phosphomolybdic acid extracted is shown in Fig. 3. Since the molybdate and ascorbate were present in excess, the amount of blue phosphomolybdate formed was limited by the number of μmoles of Pi present. When greater amounts of Pi were added at a given hydrogen ion concentration, all of the blue phosphomolybdate did not move out of the aqueous phase.

There was negligible ADP or ATP hydrolysis throughout the Pi analysis as determined by using ADP and ATP as internal standards in the procedure. The reaction supernatant fraction was slightly acid (0.18 N in perchloric acid), but it was kept cold until aliquots were removed for the addition to the butyl acetate. It could be kept for several hours at 0° C or for longer periods (12 - 24 hours) at -17° C. The Pi and organic phosphates were together only 1 1/2 minutes in the presence of the higher acidity of the ascorbate-molybdate-sulfuric acid solution. During this period no hydrolysis of ADP or ATP could be detected.

This modification of Pi analysis has both advantages and disadvantages. It is accurate to well within the margin of error of the

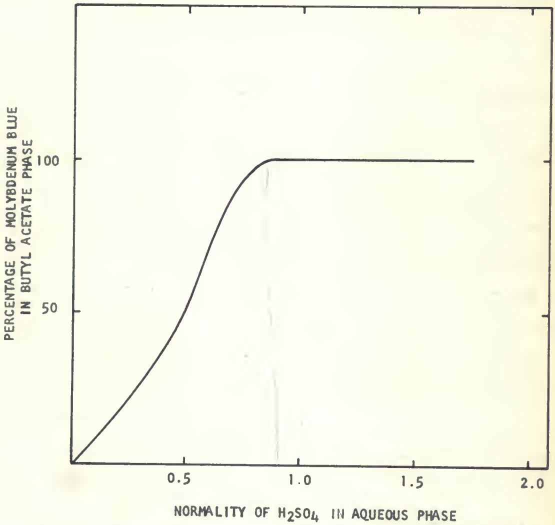

Fig. 2. The distribution of a limited amount of reduced phosphomolybdic acid between the 2 phases as influenced by acidity. The aqueous phase (total vol, 3 ml) contained 1.0 per cent ammonium molybdate, 0.2 per cent ascorbate, 0.09 μmoles Pi, and H_2SO_4 as shown. The organic phase contained 3 ml butyl acetate.

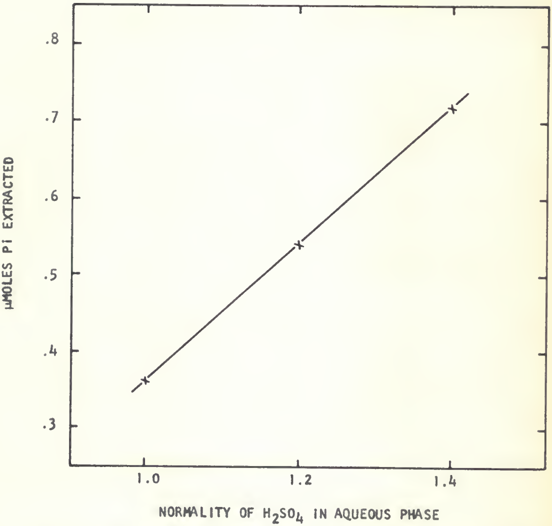

Fig. 3. The increase in the amount of Pi extracted as phosphomolybdic acid with the increase in hydrogen ion concentration. The conditions were the same as those given in Fig. 2 except that Pi concentration was varied up to 0.8 µmoles.

absorbency readings on the Spectronic 20; it does not cause hydrolysis of ADP or ATP; it eliminates much of the difficulty found with interfering substances in other procedures, although it is not as good as Berenblum and Chain's original method in this respect; and it can be used successfully when a reducing agent such as ascorbic acid is present in relatively large amounts in the material to be analyzed. On the other hand, it is more complicated than the single-phase methods and takes longer to run; it is sensitive to environmental conditions, especially hydrogen ion concentration and temperature; and it is more expensive because of the cost of butyl acetate.

ATP.--Paper chromatography was used as a qualitative analysis for the production of ATP. An aliquot of the reaction supernatant fraction was neutralized with 0.36 N potassium hydroxide and kept in the cold until the insoluble potassium perchlorate precipitated. Ten μliters per sample of the resulting supernatant fraction was spotted on a sheet of Whatman 3 MM filter paper (22 x 28 cm) with a Drummond disposable micropipette. The spots were placed 3 cm apart along one of the long edges, and a folded "pad" made from an additional piece of paper was attached to the opposite edge to permit a longer run (49). The papers were bent to form a cylinder for an ascending chromatogram, and this cylinder was placed in a jar containing 100 ml solvent. The solvent was n-propanol : 1 per cent ammonium sulfate, 2 : 1. Five μliters of a solution containing AMP, ADP, and ATP (0.025 μmoles of each) was spotted as a standard. The chromatograms were run at 22° C for 12 hours. They were dried and read with an ultraviolet lamp (Mineralight) at a wavelength of 254 mμ. Phosphate spots (Pi and nucleotide P) were occasionally checked by developing a chromatogram with the phosphate reagent described by Smith (49). ADP and ATP were clearly separated by this procedure.

PHOTOPHOSPHORYLATION IN CHLOROPLASTS FROM HEALTHY PLANTS

Before any studies could be made with chloroplasts from diseased plants, it was necessary to learn about the characteristics of "normal" chloroplasts.

The isolation of active chloroplasts depends largely upon the care with which they are extracted from the leaves. A homogenizer was used in the present experiments. Attempts to grind oat leaves with a mortar, pestle, and acid-washed sand resulted in fragmented chloroplasts which were unable to carry on phosphorylation. Since the leaves of gramineous plants have low sap and high fiber content, much more force has to be applied to them in a mortar than to succulent leaves such as spinach, which is widely used as a source of chloroplasts. It was later found that oat preparations from a mortar could be improved by increasing the coarseness of the sand, but consistently better and more uniform chloroplasts were obtained with the homogenizer. As determined by microscopic examination, preparations from the homogenizer contained a high percentage of intact chloroplasts with relatively few cell fragments. The word "intact" must be used with some reservation here, for, as Jagendorf (29) has clearly pointed out, isolated chloroplasts have obviously lost a number of soluble enzymes, cofactors, and other compounds even though they look similar to the ones *in vivo*.

It would appear that any method of extraction is suitable if it permits disruption of the cells with minimum damage to the chloroplasts. The inactivation of chloroplasts by fragmentation during extraction is not due to the loss of structural integrity, because chloroplasts which are isolated whole and then fragmented before adding to the reaction mixture can carry on phosphorylation as well as or better than comparable whole ones (10, 59). The enzymes responsible for electron transport and phosphorylation are "built into" the lamellae, the chlorophyll-containing structural units (5). Apparently the damage during extraction is at least partly caused by breaks in the limiting membrane of the chloroplast that permit injurious substances in the homogenate to contact the normally protected internal structure. The remainder of the damage is caused by losses of soluble components which are not replaced in the reaction medium.

Since practically all of the debris in the oat homogenate was removed by straining through the cheesecloth, a preliminary centrifugation was not necessary in the experiments described in this section. The sedimentation time for chloroplasts has been standardized at seven minutes at 1000 x g since Arnon (8) first isolated active preparations. With oats ten minutes at 1000 x g was required to obtain a satisfactorily complete pellet. The extra three minutes reduced the number of whole chloroplasts remaining in each ml of supernatant fraction from 1,000,000 to 300,000.

When chloroplasts are examined microscopically in a suspending solution that is approximately isotonic (0.35 - 0.40 M sodium chloride or sucrose), the ones which are not mechanically damaged are disc-shaped and smooth with distinct margins. If they are transferred to a hypotonic solution (such as the reaction medium used in the present experiments) or to distilled water, they lose their smooth, dense appearance and become swollen with indistinct and wavy margins. These changes occur quickly, although further swelling in which the limiting membrane forms a halo around the remainder of the chloroplast takes place over a longer period in a hypotonic medium.

Since sclerenchyma content increases with seedling age, the chloroplasts were more difficult to isolate as the plants became older; the best yields and the preparations most free from contamination were from seedlings seven to ten days old. Leaves of C.I. 7233, although they contain less water, were easier to extract than those of Floriland; there was less foaming during homogenization with C.I. 7233, and the chloroplast yields and rates of phosphorylation were usually higher.

Factors influencing isolation of active chloroplasts

Extracting solution.--Successful isolation of chloroplasts has been reported with sodium chloride (8) and with sucrose solutions (30). These media were compared in the first two experiments of this series. Leaves were homogenized in the 0.4 M sucrose solution described under "Materials and Methods" and in a solution of 0.35 M sodium chloride, 0.05 M Tris, and 0.02 M ascorbate. Both solutions were adjusted to pH 7.8 and phosphorylation was measured at 2400 foot-candles illumination with FMN as cofactor. The Pi uptake in µmoles per mg chlorophyll per hour was 100 for the sodium chloride treatment and 168 for the sucrose. The sucrose medium was used in all further experiments as the extracting and suspending solutions.

Air vs. nitrogen.--The excess foaming of the Floriland preparations in the homogenizer lead to the suspicion that an inactivation of some chloroplast enzymes might result from increased oxidation of the thin films. To see if anaerobic conditions during extraction would increase activity over aerobic conditions, the homogenizer cup was fitted with a rubber stopper and a piece of felt so that a steady flow of nitrogen could be passed over the extraction mixture before and during the grinding. No differences (195 μmoles Pi per mg chlorophyll per hour under air and 192 μmoles under nitrogen) were found in phosphorylation by chloroplasts isolated under the two treatments. Extractions under nitrogen were discontinued.

Hydrogen ion concentration.--Results of experiments to be described later indicated that the optimum pH for the phosphorylation reaction with FMN was approximately 8.2. Subsequently the pH of the extracting solution was also increased to 8.2 - 8.3, but the result was a striking loss of activity. To check this effect, extractions were made with solutions adjusted over a pH range of 7.0 - 9.0 at 0.5 intervals. There was little variation in activity of the preparations isolated at pH 7.0, 7.5, and 8.0, but at pH 8.5 the phosphorylation rate dropped to less than 1/3 of the others. The experiment was repeated with a narrower pH range, and the results are shown in Table 1. It can be seen that if the extracting solution is adjusted to a pH higher than 8.0 - 8.2 before homogenization, the rate of phosphorylation is sharply reduced even though the reaction itself is carried out at pH 8.2. The concentration of Tris buffer in the extracting solution permits a pH drop of 0.1 - 0.3 unit during homogenization, depending on the initial pH.

Ascorbate and stability.--When ascorbate was omitted from the extracting solution, the rates of phosphorylation in freshly prepared chloroplasts and in those after periods of storage at 0° C were decreased (Table 2).

Freezing apparently does not harm the phosphorylating ability of oat preparations. Chloroplasts stored with and without ascorbate for 24 hours at -17° C maintained most of their initial activity.

TABLE 1

RATES OF PHOSPHORYLATION IN CHLOROPLASTS EXTRACTED AT VARYING pH LEVELS

pH before extraction[a]	pH after extraction[b]	Pi uptake in µmoles/mg chl/hr
7.8	7.7	151
8.0	7.85	144
8.2	8.0	125
8.4	8.2	80
8.6	8.3	57

[a] pH adjusted in separate aliquots of extracting solution with NaOH or HCl.

[b] pH measured in first supernatant fraction.

Plants grown in greenhouse. Standard reaction mixture with FMN (0.1 µmole/ml) as cofactor, except: $MgCl_2$, 3 µmoles/ml; NaCl, 20 µmoles/ml; ADP(Na_3), 3 µmoles/ml; pH 8.2; total vol, 2 ml. Reaction time, 20 min. Illumination, 4000 f-c. Chl, 0.035 - 0.043 mg/ml, corrected to 0.04 mg.

TABLE 2

STABILITY OF CHLOROPLASTS EXTRACTED AND STORED IN SOLUTIONS WITH AND WITHOUT ASCORBATE

Storage time in hours at 0° C[a]	Pi uptake in µmoles/mg chl/hr	
	Without ascorbate[b]	With ascorbate[c]
0	210	310
3	193	310
6	239	366
24	88	276

[a] Zero time represents 45 minutes after removing chloroplasts from centrifuge. The other times are based on zero time.

[b] Extracting and suspending solutions same as those described in "Materials and Methods" except ascorbate omitted.

[c] Extracting and suspending solutions same as those described in "Materials and Methods" (0.02 M ascorbate).

Plants grown under controlled conditions. Standard reaction mixture. Reaction time, 15 min. Illumination, 4200 f-c. Chl: 0.038 mg/ml without ascorbate, 0.032 mg/ml with ascorbate.

Factors influencing photophosphorylation in chloroplasts

One of the biggest problems in making comparative studies of biological reaction rates is that of carrying out the reaction under nonlimiting conditions. The following experiments were designed to determine the optimal requirements for photosynthetic phosphorylation in oat chloroplasts under the conditions in this laboratory.

Reaction time.--Theoretically, the length of the reaction period should be limited only by the time during which phosphorylation maintains its initial linear activity under the imposed conditions. But the method of measuring ATP formation in these experiments, which is relatively insensitive as compared with the use of P^{32}, requires a period long enough to permit a large Pi uptake. As seen in Fig. 4, phosphorylation with FMN as the cofactor appeared linear for the first ten minutes. The rate in µmoles per mg chlorophyll per hour was 266 at five minutes and 268 at ten minutes. The slight break in the curve at this time, shown also in Fig. 8, indicated that a ten-minute reaction period would be the best. But with lower chlorophyll concentrations (Fig. 4, curve b), poorer preparations, or the presence of some other limiting condition, a longer period is needed to obtain sufficient uptake. Twenty minutes proved to be satisfactory, except when high chlorophyll concentrations were used or when the preparations were unusually active. In these instances, ADP became limiting (Figure 4, curve a) and the reaction stopped. Preparations did not maintain appreciable activity after 30 - 40 minutes.

Hydrogen ion concentration.--Photosynthetic phosphorylation in oat chloroplasts does not appear to have a sharp pH optimum. When PMS was used as the cofactor, maximal activity occurred around pH 7.5

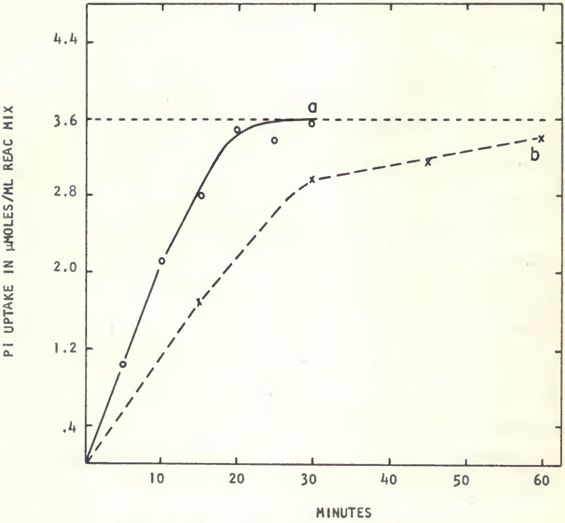

Fig. 4. Time course for photophosphorylation.

Plants grown in greenhouse. Standard reaction mixture with FMN (0.1 µmole/ml) as cofactor, except: $MgCl_2$, 3 µmoles/ml; NaCl, 1 µmole/ml; and $ADP(Na_3)$, 3.6 µmoles/ml; pH 8.3; total vol, 2 ml. Illumination, 4000 f-c. Chl: curve a, 0.048 mg/ml; curve b, 0.041 mg/ml.

The horizontal broken line shows the point at which ADP is limiting.

although no strong inhibition was evident from 7.0 - 8.0 (Fig. 5). Since Tris-hydrochloric acid does not buffer effectively below pH 7.0 or above 8.5, activities at lower or higher pH levels are difficult to measure without changing the reaction medium. When FMN was the cofactor, however, the pH optimum shifted upward to around pH 8.2.

Chlorophyll concentration.--The most suitable criterion on which to base the rate of photophosphorylation is the chlorophyll concentration in the reaction medium. The amount of chloroplasts to use in a reaction should fall within a range in which the activity increases linearly with the concentration of the chlorophyll in the medium. This range, for all practical purposes, runs from the minimum amount of chloroplasts which permits sufficient phosphorylation for accurate measurement to the amount which limits phosphorylation by not allowing light saturation under the illumination used. Therefore, the effective range, like the length of reaction time, is dependent upon the experimental conditions. Fig. 6 shows the increase in Pi uptake with increasing chlorophyll concentration in two experiments which differed only in the amount of ADP in the reaction medium and the length of the reaction. The effective working range is from about 0.025 to 0.045 mg chlorophyll per ml of reaction mixture. At concentrations over 0.1 mg chlorophyll, when PMS is used as cofactor, the amounts of ADP and Pi have to be increased in the medium and analytical troubles develop. The effect of limiting ADP is seen clearly in curve a of Fig. 6. Data from experiments such as those illustrated in Fig. 6 do not tell if the light is saturating, for illumination could be limiting over the entire range. But if the reactions in the linear range are measured at different illuminations, the slope at the lower illumination should be less if

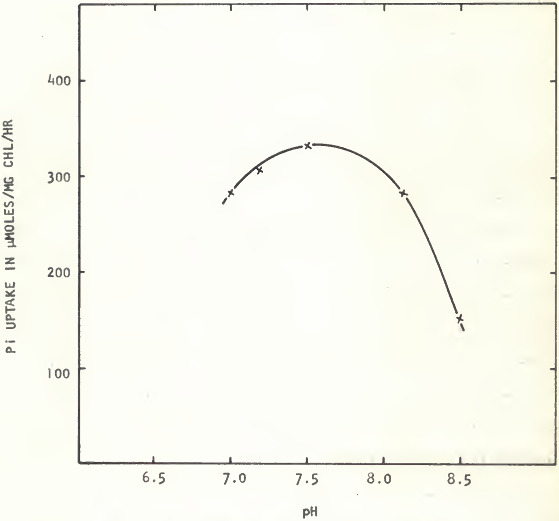

Fig. 5. pH curve for PMS-mediated phosphorylation.

Plants grown in greenhouse. Standard reaction mixture, except: $MgCl_2$, 3 μmoles/ml; NaCl, 20 μmoles/ml; total vol, 2 ml; reaction time, 15 min. Illumination, 4000 f-c. Chl, 0.028 mg/ml.

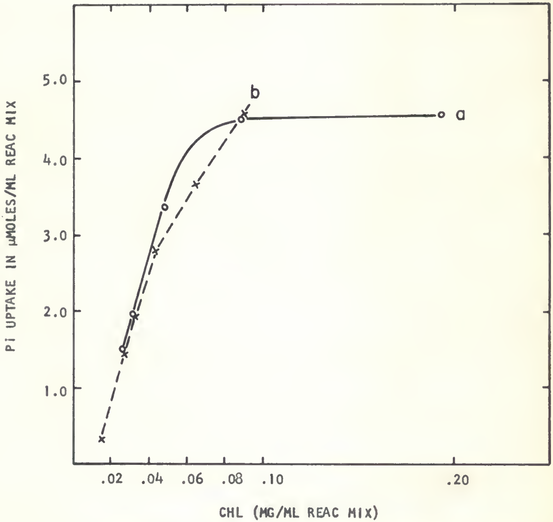

Fig. 6. Phosphorylation at varying chloroplast concentrations.

Plants grown in greenhouse.

Curve a: Standard reaction mixture, except: $MgCl_2$, 3 μmoles/ml; total vol, 2 ml. ADP, 4.6 μmoles/ml. Reaction time, 10 min. Illumination, 4000 f-c.

Curve b: Same as for a except reaction time 15 min and ADP, 5.4 μmoles/ml.

the light is not saturating. To test this, a PMS-mediated phosphorylation was conducted at two illuminations (Fig. 7). The slopes at 2800 and 4200 foot-candles were calculated to be 115 and 109 µmoles Pi per mg chlorophyll respectively, indicating a light saturation at 2800 foot-candles. The slopes of the corresponding linear portions of curves a and b in Fig. 6 were 89 and 80 µmoles per mg respectively. These lower values were not due to illumination *per se*, since they were obtained at 4000 foot-candles. But the reactions represented in Fig. 6 were carried out with 2 ml of reaction mixture, whereas those in Fig. 7 were carried out with 1 ml. The shorter light path makes it possible to obtain saturation at a lower illumination. After this series of experiments, phosphorylation was always measured in vessels containing ca. 0.04 mg chlorophyll in a total volume of 1 ml and exposed to an illumination of 2800 foot-candles.

Cofactors.--When chloroplasts are isolated in an aqueous medium, one component which they lose is their natural cofactor (or cofactors) for photophosphorylation. Although the natural cofactor is still unidentified, a large number of compounds may be added to a chloroplast preparation *in vitro* to stimulate phosphorylation.

The rates of phosphorylation with the addition of FMN, vitamin K_5, and PMS to the reaction medium are shown in Table 3. Optimal rates were reached at concentrations of 0.05 µmoles per ml and, except for FMN, these optima were maintained through a fourfold increase in concentration. Maximal rates with FMN are normally about equal to those with vitamin K_5, and the low values in the table were the result of isolating the chloroplasts at too high a pH. When FMN and vitamin K_5 were added together, the stimulation was no greater than when they

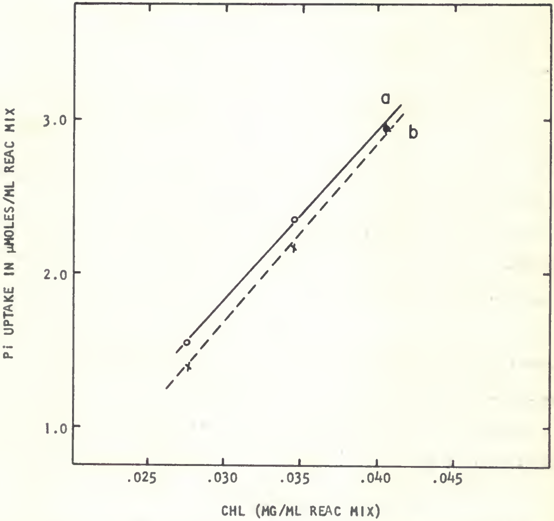

Fig. 7. Linear portion of chloroplast concentration curve at 2 illuminations.

Plants grown under controlled conditions. Standard reaction mixture, except: $MgCl_2$, 3 μmoles/ml. Total vol, 1 ml. Reaction time, 15 min.

Curve a: Illumination, 4200 f-c.

Curve b: Illumination, 2800 f-c.

TABLE 3

CONCENTRATION OF COFACTORS FOR PHOSPHORYLATION

Cofactor conc in µmoles/ml	Pi uptake in µmoles/mg chl/hr			
	FMN[a]	Vit K_5	Vit K_5 + 0.1 µmole FMN	PMS
0.005	80	27	130	68
0.01	88	55	153	100
0.05	110	173	155	302
0.1	100	170	158	302
0.2	83	167	167	313

[a] Readings lower than usual because of high pH in extracting solution.

Plants grown in greenhouse. Standard reaction mixture, except: $MgCl_2$, 3 µmoles/ml; ADP(Na_3), 3 µmoles/ml with FMN and vitamin K_5 and 6 µmoles/ml with PMS; pH 8.2; total vol, 2 ml. Reaction time, 20 min. Illumination, 4000 f-c. Chl, 0.034 - 0.044 mg/ml, corrected to 0.04 mg.

were added individually. Rates with PMS were almost double those with the other two cofactors. In another experiment, the preparations were found to be unable to carry on a measurable reaction when no cofactor was added.

Aerobic and anaerobic conditions.--It was observed that phosphorylation with FMN proceeded about as well under air as under nitrogen if the reaction time was not too long, whereas phosphorylation with PMS proceeded satisfactorily only under nitrogen. The results of two experiments designed to illustrate these differences are shown in Figs. 8 and 9. When FMN was used, aerobic and anaerobic activity ran approximately parallel for 20 - 30 minutes, after which the anaerobic activity increased. When PMS was used, aerobic activity stopped quickly at a low level and anaerobic activity continued to increase. The two experiments are not strictly comparable since the reaction with PMS was carried out in larger flasks containing a greater volume of reaction medium, thus making a longer light path. Also, anaerobiosis was interrupted when samples were removed.

Even when flasks are flushed carefully or run continuously with nitrogen, traces of oxygen remain so that a strictly anaerobic condition does not exist unless special precautions, such as trapping oxygen with catalase and ethanol, are used (24, 31). Since consistently high readings have been obtained in the present experiments with the usual flushing procedures, no attempts were made to eliminate all traces of oxygen.

Ascorbate.--When chloroplasts were incubated in the presence and absence of ascorbate, its addition unexpectedly stimulated anaerobic phosphorylation but inhibited aerobic phosphorylation (Table 4). The

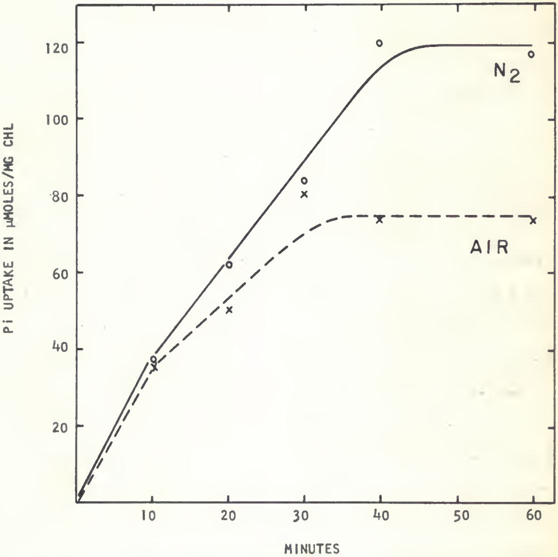

Fig. 8. FMN-mediated phosphorylation under aerobic and anaerobic conditions.

Same conditions as described in Fig. 4. Chl, 0.020 mg/ml.

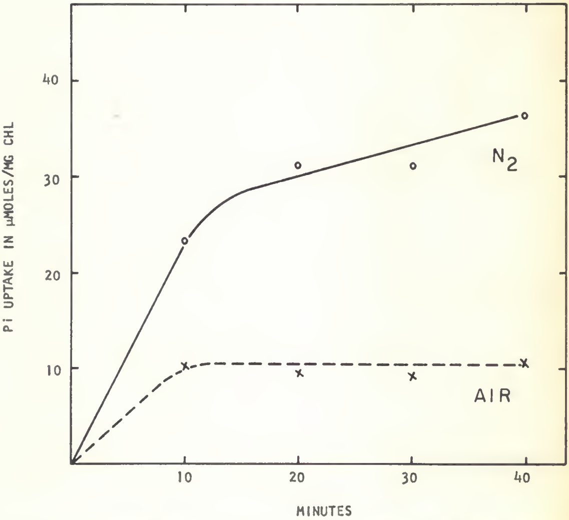

Fig. 9. PMS-mediated phosphorylation under aerobic and anaerobic conditions.

Plants grown under controlled conditions. Standard reaction mixture. Reactions run in 50 ml flasks, 6 ml per flask. 0.4 ml aliquots removed at intervals and added to an equal amount of 0.36 N $HClO_4$ in test tubes. Nitrogen run continuously in anaerobic flask except when taking samples. Illumination, 3000 f-c. Temp, 20° C. Chl, 0.037 mg/ml.

TABLE 4

PRESENCE OF REDUCTANTS IN THE REACTION MEDIUM

Treatment	Pi uptake in µmoles/mg chl/hr	
	Air	N_2
Ascorbate		
0 µmoles/ml	114	52
4 µmoles/ml	6	155
8 µmoles/ml	39	170
Glutathione		
0 µmoles/ml	105	81
4 µmoles/ml	72	318
8 µmoles/ml	34	271
Succinate, 5 µmoles/ml	64	31
Na_2SO_3, 5 µmoles/ml	(+19)	4
$(NH_4)_2SO_4$, 5 µmoles/ml	(+21)	(+16)
Control (H_2O)	78	33

A combination of 3 experiments. Plants grown under controlled conditions. In each case the chloroplasts were isolated in extracting solutions without ascorbate.

Ascorbate and glutathione experiments: Standard reaction mixture, except: no ascorbate; $MgCl_2$, 3 µmoles/ml. Reaction time, 20 min. Illumination, 2800 f-c. Chl, 0.038 mg/ml for ascorbate and 0.037 mg/ml for glutathione.

Succinate, Na_2SO_3, $(NH_4)_2SO_4$ experiment: Standard reaction mixture without ascorbate. Reaction time, 20 min. Illumination, 2800 f-c. Chl, 0.040 mg/ml.

order of treatments giving decreasing activity was as follows: nitrogen with ascorbate, air without ascorbate, nitrogen without ascorbate, and air with ascorbate. The same effect was found when ascorbate was replaced with glutathione. Succinate had no effect. Sodium sulfite and ammonium sulfate eliminated all phosphorylation and even caused a slight increase in Pi.

Leaf extract.--During the process of comparing chloroplast preparations from infected plants, portions of whole leaf extracts were added to the routinely isolated chloroplasts. The result was a consistent increase in phosphorylating activity over that of the same chloroplasts without the extracts. It became immediately apparent that components necessary for electron transport or phosphorylation were leaching out of the chloroplasts during isolation. This partly explained why some preparations had been unaccountably more active than others and why preparations from older plants were, in general, less active than those from the younger plants which were easier to extract. Since the extracts contained a large number of chloroplast fragments, there was appreciable photophosphorylation by the extracts alone; however, the combination of the extracts and the chloroplasts always gave a greater activity than the sum of their separate activities. To learn more about this stimulatory effect, other extracts and also the much more dilute supernatant fraction from the first 1000 x g sedimentation of the isolated chloroplasts were tested (Table 5). Since the supernatant fraction, with no phosphorylating capacity of its own, was as effective as the extract in stimulating the activity of the chloroplasts (previous experiments, not shown in the table), the supernatant fraction appeared to be a more satisfactory material to work with. Both extract and supernatant fraction were kept at 0° C prior to the reaction. When

TABLE 5

ADDITION OF LEAF EXTRACT AND CHLOROPLAST SUPERNATANT FRACTION TO REACTION MEDIA CONTAINING ISOLATED CHLOROPLASTS

Treatment	Pi uptake	
	µmoles/ml reac mix	µmoles/mg chl/hr
Chloroplasts alone	1.94	144
Extract alone[a]	1.76	--
Extract alone (dark)	(+0.57)	--
Chlp + heated extract[b]	2.86	212
Supernatant fraction alone[c]	(+0.31)	--
Chlp + supnt	4.13	307
Chlp + heated supnt[b]	2.95	219

[a] Extract prepared as follows: 2 g leaves, 2 ml 0.05 M Tris (pH 7.5), and sand ground together in ice-cold mortar. Homogenate squeezed through cheesecloth and centrifuged at 1000 x *g* for 15 min. Supernatant fraction saved in cold and adjusted to pH 7.5.

[b] Ten min in 100° C water bath.

[c] Supernatant fraction: refers to the supernatant fraction remaining after the first 1000 x *g* sedimentation of the chloroplasts during the normal isolation procedure with 4 g leaves and 20 ml extracting solution. Kept in cold. pH 7.4.

Plants grown under controlled conditions. Standard reaction mixture. 0.1 ml of extract or supernatant fraction added per vessel. Reaction time, 20 min. Illumination, 2800 f-c. Chl, 0.040 mg/ml.

they were heated to 100° C for ten minutes, they still maintained about half of their stimulatory power. This indicated that an unstable and a stable factor were being added to the chloroplasts. The effect of both the cold and the heated supernatant fraction was not completely lost by a twentyfold dilution (Table 6). There was essentially no reduction in activity up to a 1:5 dilution of the cold supernatant fraction and a 1:2 dilution of the heated supernatant fraction. The amount of undiluted supernatant fraction added to each reaction mixture did not contain enough Pi or chlorophyll to affect the regular measurements.

Other observations.--The reliability of the experiments reported in this paper is dependent to a great extent upon the absence of any detectable adenosine triphosphatase in the chloroplast preparations. No breakdown of ATP was observed when chloroplasts from either variety of oats were incubated in the standard phosphorylation reaction medium containing ATP instead of ADP. The results were the same whether the reaction was carried out in the light or in the dark and were confirmed by both Pi analysis and paper chromatography. A slight nonspecific phosphatase was detected (22 μmoles Pi released per mg chlorophyll per hour) when α-naphthyl phosphate was used as a substrate.

To determine if the enzyme adenylic kinase was present, once-washed oat chloroplasts routinely used in the present experiments were incubated with AMP and ATP at concentrations of 6 and 2 μmoles per ml, respectively. The Pi uptake was only 17 μmoles per mg chlorophyll per hour. This compared with a Pi uptake of 336 μmoles per mg chlorophyll per hour by the same preparation incubated with the usual amount of ADP. This slight adenylic kinase activity could also be demonstrated in chromatograms from certain experiments in which the AMP spot (a contamination

TABLE 6

DILUTION OF SUPERNATANT FRACTION ADDED TO REACTION MEDIA CONTAINING ISOLATED CHLOROPLASTS

Dilution[a]	Percentage stimulation of Pi uptake[b]	
	Cold supernatant fraction[c]	Heated supernatant fraction[c]
None	143	72
1:2	139	86
1:3	143	38
1:5	139	43
1:10	67	20
1:20	63	20

[a] Dilutions made with 0.05 M Tris (pH 7.5) and written as 1 vol of supernatant fraction made up to the desired vol of solution.

[b] For the chloroplasts alone, Pi uptake was 105 µmoles/mg chl/hr; for chloroplasts + undiluted supernatant fraction, it was 256 µmoles/mg chl/hr.

[c] Prepared as described in Table 5.

Plants grown under controlled conditions. Standard reaction mixture. 0.1 ml of designated supernatant fraction added per vessel. Reaction time, 15 min. Illumination, 2800 f-c. Chl, 0.035 mg/ml.

in the commercial ADP) was slightly larger after light or dark incubation than at zero time.

The control vessels incubated in the dark or containing heated chloroplasts (ten minutes in a water bath at 100° C) never demonstrated phosphorylation. There was usually a slight increase in Pi in the dark controls; but since the differences in readings on the spectrophotometer were often within the variations of the zero time readings, no correction was made. Hill and Walker (24) observed such an increase with spinach and Swiss chard chloroplasts and corrected for it in some of their experiments.

Discussion

Observed rates of photophosphorylation in isolated chloroplasts have steadily increased with improved techniques. The rates initially reported by Arnon *et al*. (8) were less than 5 μmoles ATP formed per mg chlorophyll per hour. Six years later Avron (10) obtained rates of 2500 μmoles ATP formed per mg chlorophyll per hour. To attain high rates it is necessary to use the lowest chlorophyll concentrations, the shortest reaction times, and the highest illuminations possible. Avron, for example, used reaction mixtures containing 0.003 mg of chlorophyll per ml incubated for two minutes at 14,300 foot-candles. Such reaction conditions are not suitable for most chloroplast studies, and the range of activity found in the present work with oats (up to 400 μmoles ATP formed per mg chlorophyll per hour) is considered quite respectable. In fact, Allen *et al*. (1) calculated that a phosphorylation rate of 500-600 μmoles per mg chlorophyll per hour is sufficient to account for the maximum carbon dioxide assimilation rate by intact leaves.

The ability of FMN, vitamin K_5, and PMS to act as redox catalysts during photophosphorylation appears to be the same with oats as it is with other preparations. The differences observed between FMN- and PMS-mediated reactions in oat chloroplasts, e.g. higher rates with PMS, different pH optima, and more inhibition with PMS under air, support the generally accepted hypothesis that FMN catalyzes a different electron transport pathway than does PMS (11, 28, 31).

There is now convincing evidence that when PMS is used in a reaction medium for photophosphorylation the active redox agent is an oxidation product, pyocyanine (24, 31). PMS is converted into pyocyanine aerobically in white light. With the normal nitrogen flushing of flasks to obtain anaerobic conditions, enough traces of oxygen remain to convert over 50 per cent of the PMS in a reaction mixture to pyocyanine. PMS alone (when its oxidation to pyocyanine is prevented) may serve as an effective cofactor for phosphorylation in spinach chloroplasts, but pyocyanine alone is more active at low concentrations (31).

The response of chloroplast preparations to aerobic and anaerobic conditions upon the addition of different cofactors has a confusing history. Contradictory results have been obtained in different laboratories and with different preparations. One misconception has been cleared up recently, however. PMS and pyocyanine are the only known cofactors which can mediate a phosphorylation under strict anaerobiosis. The other compounds, including FMN and vitamin K_3, require microamounts of oxygen (31, 53). The interpretation of this is that the reduced forms of PMS and pyocyanine are reoxidized by the primary photooxidant (a true cyclic process) whereas the reduced forms of the other cofactors are reoxidized by oxygen.

Although the photophosphorylations in the present experiments were not carried out under strict anaerobiosis, distinct differences occurred which were dependent upon the cofactor and the addition of ascorbate. The parallel activities for 30 minutes under air and nitrogen when FMN was used as the cofactor (Fig. 8) show that the reaction is initially independent of the gas phase. The later inhibition under air may be due to an accumulation of oxidized products that is prevented under nitrogen. In the absence of ascorbate, the rates of PMS-mediated phosphorylation are higher under air than under nitrogen. In the presence of ascorbate, the rates are stimulated under nitrogen and inhibited under air (Table 4, Fig. 9).

The response with FMN appears similar to that with other chloroplast preparations; however, the results with PMS are the opposite of some reports with a similar system. Avron (10), for example, found that, in the absence of ascorbate, PMS-mediated phosphorylation in Swiss chard chloroplasts was inhibited under air but not under nitrogen; the addition of ascorbate stimulated the activity under air so that the rates became equal under both phases. He concluded that ascorbate was able to substitute for nitrogen. His preparation also differed from those of oats since he found glutathione would not substitute for ascorbate. However, Jagendorf and Avron (30), using a similar system with spinach chloroplasts, found no air-nitrogen effect and no ascorbate effect. Wessels (57) and Arnon (5) both reported FMN-mediated phosphorylation in spinach chloroplasts to be about the same under air as under nitrogen, but they found different ascorbate effects. A possible explanation for some of these discrepancies is that ascorbate may be oxidized nonenzymatically and would not be available to act on the chloroplast system. Since none of the above observations was made under conditions of strict anaerobiosis, the minute requirement of FMN for oxygen would not have been influenced by the gas phase used.

The patternless results obtained with ascorbate have made it impossible to assign it a definite role in photophosphorylation. It may act in the following ways: (a) as an electron donor for a system in which ascorbate oxidation is coupled with a phosphorylation (37); (b) as a reducing agent for compounds such as FMN to prevent their reoxidation, or as a reducing agent for dissolved oxygen to prevent its combination with members of the electron transport system (54); (c) as a stabilizer which protects the system against some type of inactivation or general oxidation (5, 10); (d) as a poising agent in which catalytic amounts of ascorbate serve to maintain the proper balance between oxidation and reduction in the electron transport system (11, 25). This last possibility has been demonstrated clearly with photophosphorylation in bacterial chromatophores but no evidence exists for it with chloroplasts. Possibility (c) can be used in defense of most of the results obtained with ascorbate since it is entirely nonspecific in its application. It is used to explain the beneficial effect of adding ascorbate to the extracting solution. Possibility (b) offers a reasonable explanation for results in which the addition of ascorbate causes an increased phosphorylation under air but not under nitrogen (such as the results of Avron described above). Possibility (a) is the most satisfactory explanation for the results obtained in the present experiments because of the reverse effects under air and nitrogen. This explanation is logical if we assume that when ascorbate is added to the reaction medium it becomes the effective electron donor in the chloroplast system, whereas in its absence water is the effective electron donor. Then, with ascorbate acting as the donor, the reaction proceeds rapidly under nitrogen because the electrons flowing to the photooxidant are coupled to a phosphorylation. If, however, the preparation is incubated under air, the reaction

$$\text{Ascorbate} + 1/2\ O_2 \longrightarrow \text{dehydroascorbate} + H_2O$$

competes successfully for the electrons from ascorbate and the coupled phosphorylation is stalled. (The finding that the direct ascorbate oxidation requires at least 2 per cent oxygen (37) supports this.) When ascorbate is not present and water is acting as the donor, the presence of air does not initiate a competing reaction, and the phosphorylation is coupled to a different or partly different electron transport system. This hypothesis depends on the expectation that when chloroplasts are ripped out of their cells, dropped into a vessel containing a mixture of foreign chemicals, and shaken around for a few minutes, they may use their photolytic capacity to oxidize the most convenient material present at the time. More experimentation is obviously necessary to give this idea a respectable basis. The use of CMU, for example, would help clarify the picture since it does not inhibit ascorbate oxidation even though it strongly inhibits a concomitant phosphorylation (31). Evidence for the role of ascorbate as an effective electron donor in place of water comes from experiments by Varnon and Zaugg (55), who found that aged chloroplasts lose their ability for photoreduction and oxygen evolution but that this capacity may be restored by the addition of ascorbate plus an indophenol dye to act as an intermediate electron carrier. Also Arnon (6), by poisoning the oxygen evolution step with CMU and thereby preventing the photolysis of water, found photoreduction and phosphorylation to occur only when ascorbate was oxidized.

The abolition of photophosphorylation when ammonium sulfate was added to the reaction mixture (Table 4) was to be expected since the ammonium ion has been shown to be an effective uncoupler of phosphorylation from the electron transport to ferricyanide in spinach chloroplasts (32). The effects of the added reductants (Table 4) apparently are not correlated with their redox potentials. Ascorbate and glutathione have E_0' values of +.058 and -.340 volts, respectively. The E_0' of succinate is +.031 volts, whereas the ammonium ion is +.562 and the sulfite ion, -.454 volts. Of course, a redox correlation, if one should exist, could not be indicated until a large number of compounds had been tested.

The stimulation of photophosphorylation in isolated oat chloroplasts by the addition of heat-labile and heat-stable compounds which are lost during the leaf extraction process (Tables 5 and 6) is not surprising. Smillie (48) estimated that over 50 per cent of the soluble proteins are lost from chloroplasts during isolation in aqueous media. Vernon and Hobbs (54) reported that the photoreduction of FMN and other compounds by chloroplasts from beet leaves was dependent on a heat-labile component from the supernatant.

Since the chloroplast preparations from healthy oat seedlings were found to be satisfactory for studying reactions in the primary cycle of photosynthesis, preparations from diseased plants could be studied next. In the following section comparative measurements of photophosphorylation are described with chloroplasts from both healthy and infected leaves.

PHOTOSYNTHESIS AND INFECTION

To obtain consistently heavy infections in experimental plants, it was found necessary to inoculate the seedlings in a 10 x 15 inch flat with 40 - 60 mg of urediospores. Even under these conditions a number of leaves had to be discarded so that the material kept for homogenization would always be uniformly infected.

The chloroplasts were usually isolated from infected plants six days after inoculation. At this stage both varieties of plants show well advanced flecking; in the susceptible Floriland variety, however, the flecks are chlorotic, whereas they are necrotic in the resistant C.I. 7233 variety. With heavy infections the chlorosis or necrosis covers entire areas of the leaf. In fact, C.I. 7233 may appear much more badly damaged than Floriland because so much of the leaf tissue is dead. No uredia are visible on the surface of the leaves at this time.

Chloroplasts

The chloroplast preparations from infected plants were not badly contaminated with fungal material. The first pellet (500 x g) which was discarded during isolation contained young spores, hyphae, structures which appeared to be haustoria, host cell wall debris (particularly in C.I. 7233 preparations from heavily necrotic leaves), and the larger chloroplasts. The final preparation of chloroplasts which was used in the reaction mixture contained only occasional young spores, hyphae, and pieces of host cell walls. The structures which appeared to be haustoria were the greatest contaminant, especially in preparations from Floriland. They were not at all numerous when compared with the chloroplasts; but they were easy to see under a magnification of 430 diameters, they were about two or three times as large as the chloroplasts, and they looked almost identical to haustoria _in vivo_.

Chlorophyll to nitrogen ratio.--Individual chloroplasts from the healthy and infected leaves appeared the same when examined microscopically. To determine whether an observed reduction in chlorophyll from preparations of infected leaves represented a lower chloroplast yield or a lower chlorophyll content in the isolated chloroplasts, total nitrogen was also measured in the preparations. Six-day-old leaves from the four categories of plants were extracted routinely in 0.4 M sucrose. Total nitrogen was determined by the Hengar technique of the micro-Kjeldahl procedure and corrected for chlorophyll nitrogen. The nitrogen content was approximately the same in the preparations from healthy and infected leaves; the chlorophyll content dropped slightly in preparations from infected Floriland and as much as 37 per cent in preparations from infected C.I. 7233. Nitrogen to chlorophyll ratios calculated from two experiments were as follows: Floriland-healthy, 2.49; Floriland-infected, 2.61; C.I. 7233-healthy, 1.89; C.I. 7233-infected, 2.54. These results show that the chloroplasts isolated from the infected Floriland plants were not altered significantly in their composition, whereas at least some of the ones isolated from infected C.I. 7233 plants had lost a portion of their chlorophyll.

Rates of photophosphorylation.--The rates of photophosphorylation in chloroplasts isolated from infected plants were only slightly different from the rates of those isolated from comparable healthy plants (Table 7). No trend is apparent from the data. The maximum differences were

TABLE 7

RATES OF PHOTOPHOSPHORYLATION IN CHLOROPLASTS ISOLATED FROM HEALTHY AND RUST-INFECTED OAT SEEDLINGS

Source of chloroplasts	Pi uptake in µmoles/mg chl/hr		
	Expt 1[a]	Expt 2[b]	Expt 3[c]
Susceptible			
Floriland-healthy	218	179	90 (256)
Floriland-infected	181	221	99 (282)
Resistant			
C.I. 7233-healthy	182	158	70 (242)
C.I. 7233-infected	194	190	64 (206)

[a] From plants 16 days old, 6 days after inoculation; good infection. Standard reaction mixture, except: $MgCl_2$, 3 µmoles/ml; total vol, 2 ml. Reaction time, 20 min. Illumination, 4000 f-c. Chl in mg/ml: Floriland-healthy, 0.037; Floriland-infected, 0.036; C.I. 7233-healthy, 0.050; C.I. 7233-infected, 0.049.

[b] From plants 19 days old, 6 days after inoculation; heavy infection. Standard reaction mixture. Reaction time, 15 min. Illumination, 2800 f-c. Chl in mg/ml: Floriland-healthy, 0.037; Floriland-infected, 0.041; C.I. 7233-healthy, 0.040; C.I. 7233-infected, 0.039.

[c] From plants 19 days old, 7 days after inoculation; heavy infection. Standard reaction mixture. Reaction time, 20 min. Illumination, 2800 f-c. Chl in mg/ml: Floriland-healthy, 0.031; Floriland-infected, 0.028; C.I. 7233-healthy, 0.032; C.I. 7233-infected, 0.029. Figures in parentheses represent rates from flasks in which the chloroplasts were supplemented with supernatant fractions from healthy Floriland preparations.

approximately 40 μmoles Pi taken up per mg of chlorophyll per hour, which is within the range of variations observed between preparations of healthy Floriland.

It seemed logical at this time to try to prepare a reaction mixture for the chloroplasts as similar to the cellular environment as was possible in such an artificial system. One way to do this was to add back other fractions of the leaves together with the chloroplasts. In Table 8 are the results from a photophosphorylation system in which the isolated chloroplasts from each treatment were recombined with their respective supernatant fractions. Table 9 illustrates a system in which chloroplasts from the two infected varieties were combined with extracts from healthy and infected leaves. (The extracts differed from the supernatant fractions in that they were more concentrated and contained many more chloroplast fragments. See Tables 5 and 6 for descriptions of the preparations.) Again, activity was not significantly different in the preparations from diseased plants. The supernatant fractions and extracts apparently caused no response in photophosphorylation other than that of stimulating the rates in all of the treatments. The results in Table 9 show that the extracts from the infected plants did not contain an inhibitor of photophosphorylation. The possibility of the presence of a phosphatase in the extracts and supernatant fractions was also considered. No adenosine triphosphatase (tested as described in the last section) could be detected in the extracts from any of the categories of healthy or infected plants.

Another consideration was the effect of the parasite itself on chloroplast activity. Since the fungus can neither be grown in culture nor isolated from its host once it has become established,

TABLE 8

RATES OF PHOTOPHOSPHORYLATION IN PREPARATIONS OF CHLOROPLASTS AND SUPERNATANT FRACTIONS FROM HEALTHY AND RUST-INFECTED OAT SEEDLINGS[a]

Source of chloroplasts and supernatant fraction	Pi uptake in μmoles/mg chl/hr	
	Expt 1[b]	Expt 2[c]
Susceptible		
Floriland-healthy	305	372
Floriland-infected	338	351
Resistant		
C.I. 7233-healthy	274	277
C.I. 7233-infected	242	246

[a] Supernatant fractions prepared as described in Table 5. Each chloroplast preparation supplemented with its own supernatant fraction.

[b] From plants 17 days old, 6 days after inoculation; heavy infection. Supernatant fractions diluted 1:2 with extracting solution; 0.1 ml added per vessel. Standard reaction mixture. Reaction time, 15 min. Illumination, 2800 f-c. Chl in mg/ml: Floriland-healthy, 0.038; Floriland-infected, 0.038; C.I. 7233-healthy, 0.039; C.I. 7233-infected, 0.038.

[c] From plants 19 days old, 6 days after inoculation; heavy infection. Supernatant fraction undiluted; 0.1 ml added per vessel. Standard reaction mixture. Reaction time, 15 min. Illumination, 2800 f-c. Chl in mg/ml: Floriland-healthy, 0.037; Floriland-infected, 0.041; C.I. 7233-healthy, 0.040; C.I. 7233-infected, 0.039.

TABLE 9

RATES OF PHOTOPHOSPHORYLATION IN CHLOROPLASTS FROM RUST-INFECTED PLANTS SUPPLEMENTED WITH EXTRACTS FROM HEALTHY AND INFECTED PLANTS[a]

Source of extract	Pi uptake in µmoles/mg chl/hr[b]	
	Floriland-infected	C.I. 7233-infected
Susceptible		
Floriland-healthy	289	240
Floriland-infected	277	238
Resistant		
C.I. 7233-healthy	255	218
C.I. 7233-infected	289	212
None	166	173

[a] Extracts prepared as described in Table 5.

[b] Since the extracts alone had some phosphorylating activity, their Pi uptake was subtracted from the total uptake to give the Pi uptake of the chloroplasts.

Extracts and chloroplasts prepared from same group of plants: 15 days old, 6 days after inoculation; fair to good infection. Extracts undiluted; 0.1 ml added per vessel. Standard reaction mixture. Reaction time, 20 min. Illumination, 2800 f-c. Chl in mg/ml: Floriland-infected, 0.039; C.I. 7233-infected, 0.043.

the only way to test this effect is with the use of spores. As seen in Table 10, the rates of phosphorylation in chloroplasts isolated from healthy plants were not altered by the presence of spores or spore extracts in the reaction mixture. The urediospores were suspended in water or a dilute solution of Tween 20 for several hours prior to use in order to leach out the self-inhibitor that was present in the spores. This inhibitor prevents germination when relatively large quantities of the spores are present in small volumes of liquid (60). The wash containing the inhibitor was also added to chloroplast preparations, but the effect upon phosphorylation was no different than when the spores or the spore extracts were added. It was found that the whole spores were unsatisfactory to use with preparations of chloroplasts since they were difficult to suspend uniformly. When enough detergent (Tween 20) was added to obtain a fairly good suspension, the chloroplast activity was reduced. The concentration of Tween 20 in the reaction mixture required to inhibit phosphorylation 50 per cent (I_{50}) was 0.002 per cent.

In conclusion, rates of photosynthetic phosphorylation in isolated oat chloroplasts are not significantly influenced by crown rust infection as determined by measurements made six days after inoculation.

Whole leaves

The environmental influences upon photosynthesis and the development of infection were mentioned in the "Introduction." The effect of darkness upon the development of the rust was confirmed by germinating Floriland oats in distilled water and applying different dark and light treatments to seedlings which were inoculated when they were eight days old. No pustules developed on seedlings that were kept in the dark

TABLE 10

RATES OF PHOTOPHOSPHORYLATION IN CHLOROPLASTS FROM HEALTHY PLANTS IN THE PRESENCE OF SPORES AND SPORE EXTRACTS OF THE RUST FUNGUS

Addition to chloroplasts	Pi uptake in µmoles/mg chl/hr	
	Floriland-healthy	C.I. 7233-healthy
None	166	196
Spores[a]	161	169
None	152	128
Spore extract[b]	154	128

[a] Urediospores (30 mg) suspended in 17 ml 0.01 per cent Tween 20 for 13 hours, removed by centrifugation, and resuspended in 2 ml of the Tween 20 solution. Standard reaction mixture. Reaction time, 30 min. Illumination, 2800 f-c. Chl, 0.035 mg/ml for Floriland and 0.041 mg/ml for C.I. 7233.

[b] Urediospores (20 mg) suspended in 4 ml distilled water for 10 hours, removed by filtration, resuspended in 1 ml of 0.05 M Tris, pH 7.5, and ground in ice-cold mortar which was rinsed with 1 ml of the Tris solution. Entire homogenate used as extract. Standard reaction mixture. Reaction time, 30 min. Illumination, 2800 f-c. Chl, 0.039 mg/ml for Floriland and 0.041 mg/ml for C.I. 7233.

throughout the entire test period or on those which were in the light until inoculation and then placed in the dark. Pustules developed eight days after inoculation on seedlings that were in the light for the entire period and on those that were in the dark until inoculation and then placed in the light.

In order to determine if the effect of darkness upon the development of infection was associated with the primary or secondary cycle of photosynthesis, inhibitors were used with excised infected leaves in the light. CMU, a herbicide which is a specific and potent inhibitor of the oxygen evolution reaction (29, 58), and cyanide, an inhibitor of carbon dioxide fixation, were chosen. Cyanide does not poison the reactions of the primary cycle (photophosphorylation, oxygen evolution); but it is not specific for carbon dioxide fixation, since it is also a strong inhibitor of respiration (21). The results obtained from placing infected leaves in the light in test tubes containing solutions of both inhibitors are shown in Table II. CMU stopped the development of the rust. It did not inhibit completely at 1 ppm (5×10^{-6} M), but even at this concentration only small, occasional uredia were produced. Potassium cyanide gave irregular results, although at 100 ppm (1.5×10^{-3} M) it reduced fungal growth. This irregular response was apparently due to the failure of the cyanide to enter the cells of the host or the parasite since it acted as a better inhibitor when the solutions were acidified to pH 6.0. Glucose reversed the action of both inhibitors, but it was especially striking with CMU. The use of glucose alone, and usually of glucose plus the inhibitors, gave better sporulation than did the water control. Most of the leaves were dead seven days after they were placed in the solutions; however, the leaves in the

TABLE 11

DEVELOPMENT OF *PUCCINIA CORONATA* IN EXCISED OAT LEAVES PLACED IN SOLUTIONS OF CMU AND CYANIDE IN THE LIGHT[a]

Treatment[b]	Reaction type[c]	
	Floriland	C.I. 7233
H_2O	3 - 4	0
1% glucose	4	0
CMU, 1 ppm	0 - 1	0
CMU, 10 ppm	0	0
CMU, 10 ppm + 1% glucose	2 - 4	0
KCN, 10 ppm	1 - 4[d]	0
KCN, 100 ppm	0 - 2[d]	0
KCN, 100 ppm + 1% glucose	1 - 4[d]	0

[a] Plants same as those used for chloroplast studies. Leaves cut five days after inoculation (infection flecks beginning to show well) and left under same artificial lights. Experiment repeated 3 times with different batches of plants.

[b] Twenty ml of solution per tube; 3 leaves in each tube.

[c] Standard reaction type classification: "0" - no sporulation; "4" - heavy sporulation. Readings made 3 and 4 days after placing leaves in solutions.

[d] The lower readings were obtained when the pH of the KCN solutions was reduced to 6.0.

1 ppm CMU survived the longest and the fungus did not develop during the entire period. When spore germination tests were run with the inhibitors, as much as a 75 per cent inhibition of germination was observed with 10 ppm CMU. Neither 1 ppm CMU nor 10 and 100 ppm cyanide were inhibitory.

DISCUSSION

Phosphate metabolism and infection

Although the work reported here was primarily concerned with photophosphorylation, the original purpose was to investigate more than one aspect of phosphate metabolism in rust-infected oats. The possibility that nucleotides and phosphorylated sugars are important in host-parasite relationships is becoming a popular concept. The present trend is to explain respiratory and other metabolic changes in terms of ATP, ADP, and Pi (3, 39, 52). Critical analysis of review articles and close examination of original papers lead to the realization that such explanations are based on circumstantial evidence, insufficient experimentation, and reasoning by analogy from a few carefully proven facts.

Infection sites in susceptible plants are centers of high activity (3). Increased demands placed upon the host by the parasite are fulfilled by increased oxidations, syntheses, and transport of food materials. Accumulation of phosphate and various other substances at infection sites of obligate parasites has been demonstrated by the uptake of labeled compounds (46, 62). Whether the accumulated phosphate is free or combined has not been determined accurately. When rusted wheat seedlings were fed P^{32} through their roots, the total phosphorus of entire infected leaves was no greater than the total phosphorus of entire healthy leaves, although there was a definite accumulation around the infection zones (23). Cytological studies of rusted oat leaves showed a mycelial phosphate gradient increasing away from the infected host cells, indicating that the fungus was dependent upon the release of phosphate compounds from the host (26). High activity of acid phosphatase has been found associated with the haustoria of powdery mildew on barley (9). In the present work, an experiment was conducted with healthy and rust-infected Floriland leaves six days after inoculation in which leaf pieces were placed in vessels containing dilute phosphate buffer (pH 5.5) for 12 hours in the dark. The Pi uptake was 5 µmoles per mg total nitrogen by the healthy leaves and 17 µmoles per mg total nitrogen by the infected leaves. Although there was no way to determine how much the fungus participated in the uptake, the results support the concept of increased accumulation in infected tissue.

Failure to support the photophosphorylation hypothesis

Results of the photophosphorylation experiments conducted with chloroplasts from infected plants do not support the hypothesis presented at the first of this paper, viz. that the rust fungus would stimulate phosphorylation in chloroplasts of susceptible plants but not of resistant plants. The failure to detect responses of photophosphorylation to infection may be explained in three ways: (a) Infection may not cause changes in the primary reactions of photosynthesis *in vivo*. (b) The chloroplast preparations isolated from diseased leaves may contain relatively small numbers of chloroplasts from the infection centers and relatively large numbers from undamaged or slightly damaged cells. In a heavily infected susceptible plant, the fungus appears to be present throughout the entire tissue, and the chloroplasts in the invaded cells do not change shape or size (42). Therefore, most of the chloroplasts isolated from the infected Floriland plants in the present experiments should have come from cells in close contact with the fungus. But this may not have been true with the chloroplasts from infected C.I. 7233 plants, for in resistant varieties the chloroplasts disintegrate throughout the infection center within four or five days after inoculation (42). The increase in the nitrogen to chlorophyll ratio of preparations from leaves of C.I. 7233 indicated a loss of chlorophyll in the isolated chloroplasts; so apparently the infection centers contributed at least some of the chloroplasts in the preparations. (c) Isolated chloroplasts, even when supplemented with leaf extracts, may have been removed from chemical or physical stimulators or inhibitors that would normally affect chloroplast activity _in vivo_. This is a valid and important consideration when dealing with any cell-free preparation.

The use of isolated chloroplasts to study the progress of infection by obligate parasites offers the distinct advantage of a system in which only a host reaction is measured without any danger of participation by the parasite. But when working with chloroplasts, as with any other particulate fraction from the cell, one can never be sure what changes have occurred during isolation. Even when a standardized isolation procedure is used, the damage to the chloroplasts in one preparation may not be the same as the damage in another preparation.

Reports from the literature indicate that photosynthetic reactions are generally reduced during the course of an infection. Livne (33) found that photosynthesis (carbon dioxide fixation) began to decline in rusted wheat and bean leaves on the third day after inoculation and reached a 75 per cent reduction during sporulation. Allen (2) found oxygen evolution to decrease in the advanced stages of mildew infection on wheat. Two reports have been made on photosynthetic activities in chloroplasts isolated from virus-infected plants. Zaitlin and Jagendorf (63) measured trichlorophenol indophenol reduction, ferricyanide reduction with phosphorylation, and PMS-mediated phosphorylation in chloroplast preparations from tobacco leaves infected with tobacco mosaic virus. There were no effects upon the reactions during the first seven days of infection; but there were slight reductions in the activities during the later stages of the disease. Spikes and Stout (50) measured Hill reaction rates under varied illuminations in chloroplasts isolated from sugar beets infected with virus yellows. They found that both the photochemical and dark reactions were decreased by 50 per cent in the infected plants (time after inoculation not given).

Time of measurement

Since photosynthetic activities in the diseased plant vary with the length of the infection period, the validity of measuring photophosphorylation at the height of the flecking stage prior to sporulation (six days after inoculation) may be questioned. But from all indications this period is the one of greatest response on the part of the host. Once sporulation begins, the balance between host and parasite is lost and destruction of the host is well under way. During the flecking stage the accumulation of sugars and starch reaches a maximum (2, 27) and respiration is either at its peak or rapidly approaching it (2, 44). Therefore, if a stimulation of photophosphorylation occurs in the infected plant, it would be expected to show up during this period. Chloroplasts cannot be isolated from a heavily infected resistant variety eight days or longer after inoculation since most of the leaf tissue is dead.

Photosynthetic intermediates as a food source for parasites

Indirect evidence of a relationship between photosynthesis and infection led to Cutter's hypothesis that intermediates of carbohydrate metabolism may be required for the growth of obligate parasites. Fromme (20) and then Mains (35) were the first to demonstrate that rust development is delayed in plants that are placed in the dark during the infection period, the delay being roughly proportional to the length of time in the dark. Forward (19) expanded this work and discovered, by using 11 wheat rust differentials, that dark periods also decrease disease severity as shown by smaller pustules and more hypersensitive tissue. If, however, the plants were kept in the dark throughout the entire infection period and exposed to short intervals of light, there was no change in reaction type when two or more hours of light were used daily. Semple (45) found a more severe infection by powdery mildew in plants placed in the dark for four days immediately after inoculation but a less severe infection if the dark period was started several days after inoculation. Trelease and Trelease (51) found that if wheat plants were depleted of carbohydrates before inoculating with powdery mildew, the fungus would develop if the infected plants were placed under normal light conditions for two days before putting in the dark but not if they were in the light for only one day. Using the same host and parasite in histological studies, Pratt (41) learned that the fungus developed equally well in darkness and in light up to the third or fourth day following inoculation, but afterward there was no further development in the dark.

In 1905 (56) Ward reported that the mycelium in rust-infected plants was killed when the plants were placed under light in a carbon dioxide-free atmosphere for a few days. This has been confirmed (35) and extended (51) by showing that wheat leaves do not support mildew development in a carbon dioxide-free atmosphere unless a carbohydrate source is available.

After the discovery that light affects the development of the parasite in the host, the next logical step was to determine if carbohydrates could be substituted for light. Mains (35) found that no pustules appeared on corn leaves inoculated with rust and floated on a mineral nutrient solution in the dark although they did appear when a suitable carbohydrate was added to the solution. The Treleases (51) tested a number of mono-, di-, and polysaccharides, together with sugar alcohols at low concentrations, and found that they were all effective for the development of mildew on detached wheat leaves in the dark. When sucrose, glucose, and glycerol were applied in increasing concentrations, the fungal growth increased in proportion to the carbon content of the added materials until the osmotic pressure of the solutions became limiting. Not all sugars are suitable, as Browning (14) demonstrated by failure to get development of rust in detached oat leaves on solutions of L-arabinose, D-xylose, and D-sorbose. When he used other sugars which promoted fungal growth, the natural resistance of certain varieties of oats was broken down. This variation in reaction type on detached leaves has been observed by others (19, 51).

Successful infection of albino mutants with rust is apparently no problem as long as the plant is supplied with a carbohydrate (14, 61). But a debate exists as to whether nonfed albino leaves can support the parasite (16, 36). Cutter (16) has reported that variegated leaves of geranium do not become infected with rust except in the green portions.

The assumption that the rust parasite obtains its food normally through some intermediate of photosynthesis is strengthened by the results obtained with CMU in the present experiments. Since this inhibitor blocks one of the primary reactions of photosynthesis, it effectively stops carbon dioxide fixation and the carbon cycle. When glucose is added together with CMU, the hexose monophosphate shunt and glycolysis are stimulated, and many of the intermediate compounds that are normally produced through the carbon cycle of photosynthesis are produced from the breakdown of glucose. However, the direct effect of CMU upon the fungus, as shown by the inhibition of germination at 10 ppm, must not be overlooked. The results with cyanide were not clear enough to warrant any speculations on its value as an inhibitor of infection. Samborski and Forsyth (43) tested a number of compounds for inhibition of rust development in detached wheat leaves. They found that o-phenanthroline (which, like CMU, is an inhibitor of oxygen production in photosynthesis) was toxic to both host and fungus.

Relation of primary cycle and carbon cycle to infection

Even if it is true that a photosynthetic intermediate is required for the nutrition of an obligate parasite, the question still remains as to whether this intermediate is a product of the primary light cycle or the secondary carbon cycle of photosynthesis. Results from the present experiments and from other work suggest that the two cycles are affected differently by the parasite. The capacity of chloroplasts isolated from rust-infected leaves of susceptible plants to carry on photophosphorylation as efficiently as chloroplasts from comparable healthy leaves indicates that the primary light reactions are not affected by the parasite during the first stages of infection. But the rapid reduction in carbon dioxide fixation reported by Livne (33) for whole rusted leaves shows that the entire photosynthetic process is affected during the first stages of infection. Similar differences have been reported in photosynthetic rates of wheat leaves infected with powdary mildew. Allen (2) carried out a now classical experiment in which he measured rates of respiration and photosynthesis together with concentrations of chlorophyll, glucose, sucrose, and starch. The photosynthatic rate of leaf pieces was measured manometrically by oxygen evolution and was found to vary only slightly during the first five or six days of infection but to drop rapidly during the later stages after the parasite had reached maturity. When the rate of oxygen evolution was measured under limiting illumination and based on the amount of chlorophyll in the tissue, no reduction in activity was apparent for ten days after inoculation. Semplo (45), also using the same host and parasite, made photosynthatic measurements based on carbon dioxide fixation. His results were similar to Allen's except that he consistently found an increased activity at the time of initial sporulation before the rapid decline. Assuming that Allen's and Semplo's measurements were accurate and that the plants responded the same to infection, the discrepancy in results suggests a change in the carbon cycle of photosynthesis that was lacking in the light phase, since Allen measured oxygen evolution and Semplo measured carbon dioxide fixation. The failure of infected leaves to show a decrease in photosynthetic rates when measured under limiting illumination also indicates that the primary cycle was not affected by the disease. Shaw and Samborski (46) made radiographs from short-term light-induced $C^{14}O_2$ fixation in rust- and powdery mildew-infected leaves. Although some of their experiments showed depressions and some showed stimulations of activities at the infection sites, the conclusion was that fixation decreased in the sporulation zone and increased in the surrounding tissue.

Two explanations may be offered for the changes that occur in the carbon cycle but not in the primary light cycle of photosynthesis in plants infected with obligate parasites. (a) Intermediates of the carbon cycle may be required as a food source for the parasite, and interference with the cycle may somehow be manifest in altered rates of carbon dioxide fixation. (b) The only two known immediate products of the primary cycle, ATP and reduced triphosphopyridine nucleotide, may be used directly by the parasite or they may be used indirectly by mediating, through a non-photosynthetic pathway, the synthesis of metabolites which the parasite may require. Such a system would make the ATP and reduced triphosphopyridine nucleotide unavailable for the carbon cycle and would reduce carbon dioxide fixation, but it could not be detected by simply measuring rates of photophosphorylation.

It would be worth while to separate the primary and secondary cycles further in experiments with infected plants. Photosynthetic ratios (the amount of oxygen produced to the carbon dioxide fixed) could be obtained with whole leaves. Inhibitors such as iodoacetamide that have been shown to be specific for carbon dioxide fixation (22) would also be useful.

In view of the results on photophosphorylation presented in this paper, it is suggested that studies on intermediates of the carbon cycle in infected plants should be conducted as the next step in learning more about the relationships between photosynthesis and obligate parasitism.

SUMMARY

Chloroplasts capable of carrying on rapid rates of photosynthetic phosphorylation were isolated from seedling leaves of healthy and crown rust-infected oats.

An investigation of the characteristics of chloroplasts from healthy leaves showed that the most active and stable preparations were obtained by using an extracting solution containing sucrose and ascorbate at pH 7.5. The optimal conditions for the reaction included a reaction mixture with ascorbate, phenazine methosulfate, chloroplasts containing a total of 0.025 - 0.045 mg chlorophyll, and a pH of 7.5; a reaction time of 10 - 20 minutes; a gas phase of nitrogen; and an illumination of at least 2800 foot-candles. The supernatant fraction remaining during the isolation of the chloroplasts, especially from older leaves, contained heat-stable and heat-labile components which markedly increased rates of photophosphorylation. Flavin mononucleotide or vitamin K_5, although less effective, could be substituted for phenazine methosulfate. No adenosine triphosphatase and negligible adenylic kinase were detected in the preparations. The characteristics of oat chloroplasts were similar to those reported from other sources except that ascorbate appeared to act as an electron donor for the phenazine methosulfate-mediated phosphorylation.

A comparison of rates of photophosphorylation in chloroplasts isolated six days after inoculation from healthy and rust-infected leaves of a susceptible (Floriland) and a resistant (C.I. 7233) variety indicated that infection caused no significant differences in activity based on the chlorophyll content of the preparations. The addition of extracts from infected leaves or rust spores or spore extracts to reaction mixtures did not alter relative rates of phosphorylation. Chloroplasts from the infected resistant variety contained up to one-third less chlorophyll than those from comparable healthy plants, whereas no significant reduction was observed in the preparations from the infected susceptible plants.

Rust did not develop in excised leaves kept in the dark or in such leaves kept in the light and treated with 3-(p-chlorophenyl)-1,1-dimethylurea.

The lack of a measurable disease-induced change in photophosphorylation suggests that rust infection does not interfere with the primary light cycle in photosynthesis. However, since the development of the fungus is dependent upon photosynthesis and since carbon dioxide fixation has been reported to be reduced in rust-infected leaves, it is suggested that the rust may obtain its food supply from intermediates of the carbon cycle. A possibility also exists that the adenosine triphosphate and triphosphopyridine nucleotide produced in the primary cycle are somehow utilized by the rust.

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BIOGRAPHICAL SKETCH

Willard Kendall Wynn, Jr. was born in Raleigh, North Carolina, on March 28, 1932. He finished Broughton High School in 1950 and obtained a Bachelor of Science in Agronomy from North Carolina State College in 1955. He went into the Army for two years and finally attained the rank of private before his release in 1957. He spent a year as a graduate assistant in the Department of Plant Pathology at Cornell University and then taught biology for a year at Brewton-Parker Junior College, Mt. Vernon, Georgia. He entered the Graduate School at the University of Florida in September, 1959, and held a teaching assistantship until he finished his graduate work in April, 1963.

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

April 20, 1963

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